



## Environmental Microbiology

Antibiotic resistance genes detected in the marine sponge *Petromica citrina* from Brazilian coast

Marinella Silva Laport<sup>a,b,\*</sup>, Paula Veronesi Marinho Pontes<sup>a</sup>, Daniela Silva dos Santos<sup>a</sup>, Juliana de Fátima Santos-Gandelman<sup>a</sup>, Guilherme Muricy<sup>c</sup>, Mathieu Bauwens<sup>b</sup>, Marcia Giambiagi-deMarval<sup>a</sup>, Isabelle George<sup>b</sup>

<sup>a</sup> Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro (UFRJ), Cidade Universitária, Rio de Janeiro, RJ, Brazil

<sup>b</sup> Département de Biologie des Organismes, Laboratoire de Biologie Marine, Université Libre de Bruxelles (ULB), Bruxelles, Belgium

<sup>c</sup> Museu Nacional, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil

## ARTICLE INFO

## Article history:

Received 21 July 2015

Accepted 11 January 2016

Available online 21 April 2016

Associate Editor: John Anthony McCulloch

## Keywords:

Microbes

Natural hotspots

Porifera

Resistance

Rio de Janeiro

## ABSTRACT

Although antibiotic-resistant pathogens pose a significant threat to human health, the environmental reservoirs of the resistance determinants are still poorly understood. This study reports the detection of resistance genes (*ermB*, *mecA*, *mupA*, *qnrA*, *qnrB* and *tetL*) to antibiotics among certain culturable and unculturable bacteria associated with the marine sponge *Petromica citrina*. The antimicrobial activities elicited by *P. citrina* and its associated bacteria are also described. The results indicate that the marine environment could play an important role in the development of antibiotic resistance and the dissemination of resistance genes among bacteria.

© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

The spread of antibiotic-resistant microorganisms in the environment is globally recognized as an important public health issue, and there are concerns on our future ability to treat infectious diseases.<sup>1</sup> Therefore, the knowledge of the nature of these resistance determinants in natural habitats

is indispensable to get a better insight of the development of antibiotic resistance in clinical settings.<sup>2</sup>

In a previous publication, Marinho and colleagues<sup>3</sup> demonstrated the antimicrobial and cytotoxic activities of the compound halistanol trisulphate isolated from *P. citrina*. This compound exhibited a broad-spectrum antibacterial activity against certain medically important bacteria, including resistant strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*,

\* Corresponding author.

E-mail: [marinella@micro.ufrj.br](mailto:marinella@micro.ufrj.br) (M.S. Laport).

<http://dx.doi.org/10.1016/j.bjm.2016.04.016>

1517-8382/© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

*Enterococcus faecalis*, *Mycobacterium fortuitum* and *Neisseria gonorrhoeae*.<sup>3</sup>

Symbiotic microbial communities can significantly impact the host-sponge ecology and evolution through supplemental nutrition and by the production of bioactive substances that can deter predators, competitors, and fouling organisms. Many of these substances possess antibacterial activity.<sup>4</sup> The microbes that produce these antibiotics harbor resistance genes to protect themselves. Therefore, the selective pressure of the environment shapes these bacterial communities.<sup>5</sup>

In this background, the aim of the present study was to detect the resistance genes in culturable and unculturable bacteria associated with the sponge *P. citrina*. This study is the first report detecting the antibiotic resistance genes in *P. citrina* by culture-independent approaches. Such genes have usually been described in pathogenic bacteria.

## Material and methods

### Sponge collection and bacteria used in this study

The samples of the sponge *P. citrina* were collected by scuba-diving at a depth of 4–20 m at Cagarras Archipelago (23801'S–43811'W), located in Rio de Janeiro, south-eastern Brazil (south-western Atlantic).

The bacterial strains were isolated and identified from *P. citrina* by Santos-Gandelman and colleagues in an earlier study.<sup>6</sup> Of them, six were selected according to their antibacterial activity against certain medically important strains<sup>7</sup> and/or antibiotic resistance profile.<sup>6</sup> *Bacillus* Pc31 and Pc32, *Enterococcus* Pc5b and *Shigella* Pc5a strains were grown in brain–heart infusion medium (BHI) (Difco, MI, USA), and *Bacillus* Pc3M and *Halomonas* Pc51M were grown in a marine medium (Marine 2216, Difco), at 25 °C for 24 h.

The following strains were included as positive controls for specific amplification of the different genes under investigation: *Escherichia coli* LO (*qnrA*), *E. coli* EB2b (*qnrB*), *Streptococcus agalactiae* (*ermB*), *S. agalactiae* CL5596 (*tetL*), *Staphylococcus haemolyticus* MD2 (*mecA* and *mupA*). These strains were grown in BHI medium at 37 °C for 18 h.

### Polymerase chain reaction amplification

DNA from 0.25 g of the sponge body was extracted using the Ultra Clean Soil DNA isolation kit (Mo Bio, Carlsbad, CA, USA) following the manufacturer's protocol. DNA from the bacterial strains was isolated by the guanidinium thiocyanate extraction method.<sup>8</sup>

Thus, the total DNA isolated from the bacteria from the sponge samples and from the culturable bacteria isolated from *P. citrina* were used to amplify genes conferring resistance to macrolide-lincosamide-streptogramin (*ermB*), methicillin (*mecA*), mupirocin (*mupA*), quinolones (*qnrA*, *qnrB*), and tetracyclines (*tetL*).

The following primers were used: for *ermB*, F: 5-CATTT-AACGACGAACTGGC and R: 5-GGAACATCTGTGGTATGGCG,<sup>9</sup> to give a 425-bp product; for *mecA*, F: 5-TAGAAATGACTGAA-CGTCCG and R: 5-TTGCGATCAATGTTACCTAG,<sup>10</sup> to give a 154-bp product; for *mupA* F: 5-GTTTATCTTCTGATGCTGAG

and R: 5-CCCCAGTTACACCGATATAA,<sup>11</sup> to give a 237-bp product; for *qnrA*, F: 5-ATTTCTCACGCCAGGATTG and R: 5-GATCGGCAAAGGTTAGGTCA,<sup>12</sup> to give a 516-bp product; for *qnrB*, F: 5-GATCGTGAAAGCCAGAAAGG and R: 5-ACGATGCCTGGTAGTTGTCC,<sup>12</sup> to give a 469-bp product; for *tetL*, F: 5-ATAAATTGTTTCGGGTCGGTAAT and R: 5-AACCA-GCCAACTAATGACAATGAT,<sup>13</sup> to give a 1077-bp product.

The reaction mixtures, in final volumes of 50 µL, contained MgCl<sub>2</sub> (1.5 mM for the *mecA* and *mupA* genes; 2 mM for the *ermB* and *tetL* genes, and 4 mM for the *qnrA* and *qnrB* genes), deoxynucleoside triphosphates (0.2 mM each), primers (0.5 µM each), Taq DNA polymerase (0.5 U), reaction buffer (10 mM), and 10–20 ng of the extracted DNA as the template.

The PCR conditions were initial denaturation at 94 °C for 5 min, followed by 32 cycles at 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 60 s, with a final elongation step at 72 °C for 5 min.<sup>12</sup> The positive (strains with known resistance genes) and negative (without DNA template) controls were included in each run. Amplification products were provisionally identified from their sizes in ethidium bromide-stained agarose gels.

## Results and discussion

The information about the selection pressures on antibiotic resistance genes is very limited regarding the remote environments with low direct human contacts. A more comprehensive understanding of the natural roles of putative antibiotic resistance genes is crucial in understanding of their origin and functions.<sup>14</sup>

In recent years, several antibiotics and other bioactive molecules have been isolated from marine sponges<sup>15</sup> and from sponge-associated bacteria,<sup>4,16</sup> including *P. citrina*<sup>3</sup> and its associated bacteria.<sup>7</sup>

The *P. citrina* samples were collected at Cagarras Archipelago, which is a recent marine protected area located on the coast of Rio de Janeiro, Brazil. These islands are impacted both by the Guanabara Bay waters and by the discharges from a submarine outfall that releases untreated domestic sewage, both of which are balanced by the influx of pristine offshore water masses.<sup>17</sup>

In this study, resistance genes for different antibiotics were detected in the DNA extracted from the culturable and unculturable bacteria associated with the sponge *P. citrina*. All amplicons were of the sizes of those of the positive controls (Table 1). The antibiotic resistance profile of the culturable bacteria associated with *P. citrina* has already been reported.<sup>6</sup> This conforms to the data reported herein, as we have reported genes for quinolone and erythromycin resistance. Besides, the results also indicate that the hologenome of *P. citrina* contains genes encoding antibiotic resistance to erythromycin, methicillin, mupirocin, quinolone, and tetracycline. This goes in line with the fact that many marine sponges harbor dense and diverse microbial communities of considerable ecological and biotechnological importance.<sup>5</sup>

The application of culture-independent approaches, such as PCR and metagenomics, for the study of antibiotic resistance genes in the environment has uncovered a vast diversity of antibiotic resistance genes in soil bacteria. However, according to the best of our knowledge, this is the first time that

**Table 1 – Antibiotic resistance genes detected in the culturable and unculturable bacteria associated with the sponge *P. citrina*.**

Resistance genes	<i>qnrA</i>	<i>qnrB</i>	<i>ermB</i>	<i>tetL</i>	<i>mecA</i>	<i>mupA</i>
DNA template						
Sponge	–	+	–	+	+	+
<i>Bacillus</i> Pc31	+	+	+	–	–	–
<i>Bacillus</i> Pc32 (SXT <sup>R</sup> )	+	+	+	–	–	–
<i>Bacillus</i> Pc3M (CIP <sup>R</sup> , GEN <sup>R</sup> , SXT <sup>R</sup> )	–	+	–	–	–	–
<i>Enterococcus</i> Pc5b (GEN <sup>R</sup> )	–	+	–	–	–	–
<i>Halomonas</i> Pc51M (ATM <sup>R</sup> , CAZ <sup>R</sup> , CFE <sup>R</sup> )	–	–	+	–	–	–
<i>Shigella</i> Pc5a (SXT <sup>R</sup> , TET <sup>R</sup> )	–	–	+	+	–	–
Positive controls <sup>**</sup> (amplicon size – bp)	516	469	425	1077	154	237

\* Antibiotic resistance to: aztreonam (ATM<sup>R</sup>), ceftazidime (CAZ<sup>R</sup>), cephalexin (CFE<sup>R</sup>), ciprofloxacin (CIP<sup>R</sup>), gentamicin (GEN<sup>R</sup>), trimethoprim/sulfamethoxazole (SXT<sup>R</sup>), tetracycline (TET<sup>R</sup>) (Santos-Gandelman et al.<sup>6</sup>).

\*\* Positive control strains (DNA template): *qnrA*, *Escherichia coli* LO; *qnrB*, *Escherichia coli* EB2; *ermB*, *Streptococcus agalactiae*; *tetL*, *Streptococcus agalactiae* CL 5596; *mecA* and *mupA*, *Staphylococcus haemolyticus* MD2. Amplicon detection: +, positive; –, negative.

*mecA*, *mupA*, *qnrB* and *tetL* were detected in sponge-associated bacteria by culture-independent approaches. These results demonstrate that PCR is also a powerful tool to detect potential antibiotic resistance genes in marine environments.

While many antibiotic-resistance genes are believed to have their origin in natural ecosystems, their abundance, nature, and ecological role in such settings remain relatively obscure. In addition, antibiotics used in therapeutics and agriculture are known to accumulate in the environment and to contaminate aquatic habitats, where they can exert a selective pressure on the native flora.<sup>18</sup> Erythromycin, quinolone and tetracycline-resistant bacteria can be found even in pristine environments and in animals.<sup>19</sup> Recently, some of these resistance genes have been identified in a *Bacillus* sp. isolated from the sponge *Haliclona simulans*.<sup>20</sup> The plasmid-mediated quinolone resistance genes, *qnrA* and *qnrB*, have already been detected in bacterial strains isolated from aquatic environments.<sup>21</sup> These genes are horizontally transferable among bacteria.<sup>18</sup>

Mupirocin, which is also known as pseudomonic acid A, is produced by *Pseudomonas fluorescens* isolated from soil environments.<sup>21</sup> Plasmids that confer high-level resistance to mupirocin were isolated nearly twenty years before the clinical use of this drug.<sup>22</sup> The *mupA* gene was also found in the bacterium, *Oceanobacillus iheyensis*, isolated from deep-sea sediments at a depth of over 1000 m.<sup>23</sup>

The *mecA* gene is usually acquired along with a variety of genetic elements. The origin of *mecA* and the other genes of these cassettes have been the subject of an intensive research since the original discovery of methicillin-resistant *Staphylococcus aureus* (MRSA).<sup>24</sup> Earlier works suggested homology with *mec* genes found in the coagulase-negative *Staphylococcus sciuri* group, which has been isolated from animals and food products, and occasionally from humans.<sup>25,26</sup> Other authors speculated that *mecA* originated from *Staphylococcus fleurettii*, a species isolated from raw-milk cheese.<sup>27</sup> While it has been reported that some antibiotic resistance genes might have originated from marine bacteria, it is probably not the case of *mecA*, as the relationship of *Staphylococcus* with the marine environment remains elusive.<sup>28</sup>

## Conclusions

It is important to characterize the resistance genes in the entire marine community, including both culturable and unculturable strains.<sup>18</sup> Little is known about the antibiotic resistome of the vast majority of the environmental bacteria, although there have been calls for a better understanding of the environmental reservoirs of antibiotic resistance and their potential impacts on clinically important bacteria.<sup>29</sup> The prevalence and diversity of the resistance genes in the environment inspire hypotheses about the native roles of these resistance genes in the natural microbial communities. Considering that antibiotic treatment is our primary, and in many cases only, method of treating infectious diseases, we conclude that more detail studies of the environmental reservoirs of the resistance genes are crucial for our ability to fight infections in future.

## Funding

This work was supported by grants from CAPES, CNPq and FAPERJ to M.S. Laport. D.S. Santos, J.F. Santos-Gandelman and P.V.M. Pontes received FAPERJ, CAPES and CNPq fellowships, respectively.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

The authors give special thanks to Dr. Walter Oelemann for his assistance in the preparation of this manuscript and to Dr. Kátia Regina Netto dos Santos, Dr. Lucia Martins Teixeira and Dr. Renata Picão for providing bacterial strains used as the controls in the PCR reactions.

## REFERENCES

- Vignaroli C, Luna GM, Rinaldi C, Di Cesare A, Danovaro R, Biavasco F. New sequence types and multidrug resistance among pathogenic *Escherichia coli* isolates from coastal marine sediments. *Appl Environ Microbiol.* 2012;178:3916–3922.
- Phelan RW, Clarke C, Morrissey JP, Dobson AD, O'Gara F, Barbosa TM. Tetracycline resistance-encoding plasmid from *Bacillus* sp. strain #24, isolated from the marine sponge *Haliclona simulans*. *Appl Environ Microbiol.* 2011;77:327–329.
- Marinho PR, Simas NK, Kuster RM, Duarte RS, Fracalanza SE, Ferreira DF, Romanos MT, Muricy G, Giambiagi-deMarval M, Laport MS. Antibacterial activity and cytotoxicity analysis of halistanol trisulphate from marine sponge *Petromica citrina*. *J Antimicrob Chemother.* 2012;67:2396–2400.
- Santos-Gandelman JF, Giambiagi-deMarval M, Oelemann WMR, Laport MS. Biotechnological potential of sponge-associated bacteria. *Curr Pharm Biotechnol.* 2014;10:86–105.
- Webster NS, Taylor MW. Marine sponges and their microbial symbionts: love and other relationships. *Environ Microbiol.* 2012;14:335–346.
- Santos-Gandelman JF, Santos OCS, Pontes PVM, Andrade CL, Korenblum E, Muricy G, Giambiagi-deMarval M, Laport MS. Characterization of cultivable bacteria from Brazilian sponges. *Mar Biotechnol.* 2013;15:668–676.
- Santos OCS, Pontes PVM, Santos JFM, Muricy G, Giambiagi-deMarval M, Laport MS. Isolation, characterization and phylogeny of sponge-associated bacteria with antimicrobial activities from Brazil. *Res Microbiol.* 2010;161:604–612.
- Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol.* 1989;8:151–156.
- Jensen LB, Frimodt-Møller N, Aarestrup FM. Presence of *erm* gene classes in Gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiol Lett.* 1999;170:151–158.
- Del Vecchio VG, Petroziello JM, Gress MJ, McCleskey FK, Melcher GP, Crouch HK, Lupski JR. Molecular genotyping of methicillin-resistant *Staphylococcus aureus* via fluorophore-enhanced repetitive-sequence PCR. *J Clin Microbiol.* 1995;33:2141–2144.
- Nunes EL, Santos KRN, Mondino PJ, Bastos MC, Giambiagi-deMarval M. Detection of *ileS-2* gene encoding mupirocin resistance in methicillin-resistant *Staphylococcus aureus* by multiplex PCR. *Diagn Microbiol Infect Dis.* 1999;34:77–81.
- Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA, Hooper DC. *qnr* prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. *Antimicrob Agents Chemother.* 2006;50:2872–2874.
- Duarte RS, Barros RR, Facklam RR, Teixeira LM. Phenotypic and genotypic characteristics of *Streptococcus porcinus* isolated from human sources. *J Clin Microbiol.* 2005;43:4592–4601.
- Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol.* 2010;8:251–259.
- Laport MS, Santos OCS, Muricy G. Marine sponges: potential sources of new antimicrobial drugs. *Curr Pharm Biotechnol.* 2009;10:86–105.
- Santos OCS, Soares AR, Machado FLS, Romanos MTV, Muricy G, Giambiagi-deMarval M, Laport MS. Investigation of biotechnological potential of sponge-associated bacteria collected in Brazilian coast. *Lett Appl Microbiol.* 2015;60:140–147.
- Batista D, Muricy G, Rocha RC, Miekeley NF. Marine sponges with contrasting life histories can be complementary biomonitoring of heavy metal pollution in coastal ecosystems. *Environ Sci Pollut Res Int.* 2014;21:5785–5794.
- Aminov RI. The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol.* 2009;11:2970–2988.
- Suzuki S, Hoa PT. Distribution of quinolones, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in Indochina. *Front Microbiol.* 2012;22:3–67.
- Barbosa TM, Phelan RW, Leong D, Morrissey JP, Adams C, Dobson AD, O'Gara F. A novel erythromycin resistance plasmid from *Bacillus* sp. strain HS24, isolated from the marine sponge *Haliclona simulans*. *PLoS One.* 2014;9:e115583.
- Sutherland R, Boon RJ, Griffin KE, Masters PJ, Slocombe B, White AR. Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. *Antimicrob Agents Chemother.* 1985;27:495–498.
- Takasu H, Suzuki S, Reungsang A, Pham HV. Fluoroquinolone (FQ) contamination does not correlate with occurrence of FQ-resistant bacteria in aquatic environments of Vietnam and Thailand. *Microbes Environ.* 2011;26:135–143.
- Rahman M, Connolly S, Noble WC, Cookson B, Phillips I. Diversity of staphylococci exhibiting high-level resistance to mupirocin. *J Med Microbiol.* 1990;33:97–100.
- Lu J, Nogi Y, Takami H. *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. *FEMS Microbiol Lett.* 2001;205:291–297.
- Hiramatsu K, Cui L, Kuroda M, Ito T. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol.* 2001;9:486–493.
- Antignac A, Tomasz A. Reconstruction of the phenotypes of methicillin-resistant *Staphylococcus aureus* by replacement of the staphylococcal cassette chromosome *mec* with a plasmid-borne copy of *Staphylococcus sciuri* *pbpD* gene. *Antimicrob Agents Chemother.* 2009;53:435–441.
- Couto I, de Lencastre H, Severina E. Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri*. *Microb Drug Resist.* 1996;2:377–391.
- Vernozy-Rozand C, Mazuy C, Meugnier H, Bes M, Lasne Y, Fiedler F, Etienne J, Freney J. *Staphylococcus fleurettii* sp. nov., isolated from goat's milk cheeses. *Int. J. Syst Evol Microbiol.* 2000;4:1521–1527.
- Moellering RC Jr. MRSA: the first half century. *J Antimicrob Chemother.* 2012;67:4–11.
- Davies J, Davies D. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev.* 2010;74:417–433.